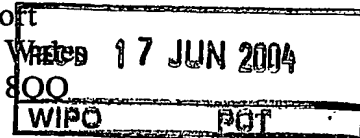




INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8OO



I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Stephen Handley

Dated 4 June 2004

**PRIORITY
DOCUMENT**
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

28 MAR 2003

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference

28 MAR 2003

IS/BP6125272

2. Patent application number

(The Patent Office will fill in this part)

0307232.9

3. Full name, address and postcode of the or of each applicant (underline all surnames)

ZYLEPSIS LIMITED
6 Highpoint
Henwood Business Estate
Ashford
Kent, TN24 8DH

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

6575575002

4. Title of the invention

Production of Vanillin

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP

Patents ADP number (if you know it)

109006 ✓

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number
(if you know it)

Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

Yes

- a) any applicant named in part 3 is not an inventor, or
 - b) there is an inventor who is not named as an applicant, or
 - c) any named applicant is a corporate body.
- See note (d))

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form.
Do not count copies of the same document

Continuation sheets of this form

Description

13 ✓

Claim(s)

Abstract

Drawing(s)

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

Request for substantive examination (*Patents Form 10/77*)

Any other documents
(please specify)

11. I/We request the grant of a patent on the basis of this application.

Signature

Date
27 March 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

Ian Stuart

0117 9266 411

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

PRODUCTION OF VANILLIN

The present invention relates to methods and materials that may be used in the production of vanillin.

5 There is a demand for natural vanillin of high flavour quality. This requires the use of raw materials of natural origin, and that all process steps should be compatible with the requirements for natural flavours as recognised by the industry and regulatory authorities.

10 It is known to convert ferulic acid into vanillin using various microorganisms. We have previously disclosed (WO 00/50622) that use of a special strain of *Pseudomonas putida* can lead to a culture medium containing 2.25 gl^{-1} of vanillin, a molar yield of 75%
15 based on ferulic acid consumed. Vanillin was recovered by separating the culture broth from the cells and extracting it with an organic solvent (butyl acetate).

 Haarman & Reimer GmbH have disclosed (US-A-6133003) two strains of *Amycolatopsis*. Using one of them they
20 achieved a culture medium containing up to 11.5 gl^{-1} of vanillin and 1 gl^{-1} of unreacted ferulic acid. These concentrations were determined by HPLC. There is no disclosure of any work-up technique or the isolation of the product.

Givaudan-Roure (International) S.A. have disclosed (EP-A-0885968) the use of *Streptomyces setonii* to produce "vanillin and several by-products". The concentration of vanillin is said to be 8-16 gl^{-1} , though in the actual
5 examples it ranges from 3.10 to 13.9 gl^{-1} , in the latter case accompanied by 0.4 gl^{-1} of guaiacol. The co-production of guaiacol is represented as being advantageous. The products are extracted by solvent extraction using methyl tert-butyl ether.

10 In many fields, the use of organic solvents is now seen as undesirable. This is certainly the case in the production of food-grade materials, especially if they are to be of "organic" quality.

We have now developed a process for the production
15 of vanillin which does not require the use of organic solvents. Independent aspects of the invention include:

(a) novel microorganisms (Zyl 926, and mutants thereof) for converting ferulic acid into vanillin;

(b) a microbiological process for preparing a
20 culture medium containing vanillin as the predominant solute, preferably using the microorganism (a);

(c) a process for obtaining vanillin from a solution (particularly one derived from the culture medium (b)) comprising:

25 (i) precipitating solid crude vanillin; and

(ii) purifying the crude vanillin by high pressure liquid CO₂ extraction or (less preferably) another form of extraction using CO₂ (supercritical or porocritical extraction), or short path distillation; and/or by fluidised bed drying, preferably using CO₂ as the fluidising gas;

(d) natural/organic vanillin of high flavour quality as produced by the above process.

10 a) Novel microorganism

Zyl 926 is a new organism which we have produced. It has been deposited with CABI Bioscience, Egham TW20 9TY, GB and given the accession number IMI 390106. It has been identified as a strain of *Amycolatopsis*. It has the following characteristics:-

(i) Resistance to spectinomycin (determined by plating strains onto ISP II agar (see below) containing 1g/l spectinomycin, incubating, and observing the growth or non/growth of colonies);

20 (ii) Morphology:

Media: ISP II medium, 5 day evaluation

Form: Irregular

Elevation: Raised

Margin: Curled/undulate

25 Colony Colour: Yellow/beige

Spore Colour: White

Surface: Dull, presporulation

The criteria for selection of the strain were that

5 it be:-

- Genetically stable.
- Safe (Class 1).
- Easy to grow to high biomass concentrations using a cheap carbon source.
- 10 ➤ Produce concentrations of vanillin significantly higher than existing strains.
- Convert nearly all the ferulic acid supplied.
- Carry out the conversion in an acceptable time period.
- 15 ➤ Produce no significant off-flavour by-products, or by-products that colour the product.

b) Microbiological Process

An organism having a known antibiotic resistance
20 (preferably Zyl 926) is incubated in the presence of the antibiotic to prepare a stock of the organism, essentially free of other organisms. Subsequent processes use this stock material, without added antibiotic.

A sterile medium is inoculated with the organism, which is cultured. Ferulic acid is added to the cultured organism, and undergoes bioconversion to vanillin. The broth, containing the vanillin in solution, is removed
5 and clarified.

c) Downstream Purification

An aqueous solution of vanillin (generally the clarified broth from (b)) is treated (typically by
10 concentration and cooling) to cause solid vanillin to separate. This is collected and purified, preferably by extraction with liquid CO₂. Extraction with liquid carbon dioxide is favoured because of its good selectivity, ability to deal with variations in feedstock, operation
15 at low temperature and good solvating power. Extraction efficiencies of 95% have been achieved to yield a product with a vanillin content of 98-100%. This product may be milled to aid rapid dissolution in formulation applications. The resulting material may be further
20 purified by careful washing with a solvent (preferably one acceptable for use in a process for making natural flavours) to remove the surface material, where impurities tend to be concentrated. Alternatively or additionally, further purification can be achieved by
25 fluidised bed "drying" to remove volatile impurities.

Indeed this could be used for the primary purification of the separated solid vanillin.

This form of purification, involving the direct production of solid crude vanillin, requires a starting
5 solution which contains predominantly vanillin, with only small amounts of impurities and unreacted ferulic acid. In particular, odoriferous contaminants (such as guaiacol) are very undesirable. Desirably the concentration of vanillin in a broth from a
10 microbiological process should be at least 5g l^{-1} and preferably at least 10g l^{-1} . The broth obtained from process (b) using Zyl 926 is particularly suitable.

(d) Natural/Organic Vanillin

15 The vanillin which we can produce can meet the following criteria:

(i) Vanillin content of $\geq 98\%$ w/w.

(ii) No odorous impurity (off-aroma) such as guaiacol, vinylguaiacol, eugenol, isoeugenol present at
20 more than ca 100ppm in the solid vanillin product.

(iii) Colour. A 'L' value of ≥ 94 determined using a Minolta chromameter calibrated with propylene glycol and using samples of 6% vanillin in propylene glycol.

(iv) Isotope ratio consistent with the ferulic acid
25 precursor having been entirely derived from a natural

plant source (e.g. maize, sugar beet or rice) as defined by authorities such as the French Ministere de L'Economie des Finances et de L'Industrie (59, Bd Vincent Auriol, 75703, Paris, Cedex 13, France).

5 Some embodiments of the invention will now be described in greater detail by way of example.

1. Preparation of stock inoculum

10 Petri dishes containing sterile ISP II agar were inoculated aseptically with Zyl 926 culture. (ISP II agar contains 10g/L malt extract, 4gl⁻¹ yeast extract, 4gl⁻¹ glucose and 20gl⁻¹ agar. This is adjusted to pH 7.4, and 1gl⁻¹ spectinomycin is added as the agar cools (and before it gels). These petri dishes were incubated for a
15 minimum of 72 hours at 30°C to generate colonies across the plates.

 Single colonies were lifted from the agar and used to inoculate 50ml portions of sterile SFG medium containing 1gl⁻¹ spectinomycin in 250ml Erlenmeyer flasks.
20 The antibiotic was added by sterile filtration. (SFG medium: per litre of water: Soya flour (Nutrisoy) 5g; dipotassium hydrogen orthophosphate 1g; magnesium sulphate heptahydrate 1.64g; glycerol 20g). Flasks were incubated at 30°C and 200rpm for 3 to 5 days in a shaking incubator.

After visual assessment of satisfactory luxuriant growth an equal volume of sterile cryopreservative was added: 20% glycerol / 10% lactose solution in water.

5 The resulting suspension of cells was then dispensed into sterile vials in appropriate aliquots for inoculation of subsequent stages. Culture stock was preserved by storage at -80°C .

2. Fermentation

10 0.7ml of culture (thawed) prepared as above was used to inoculate 1 litre of sterile SFG medium in a 1.25l fermenter, configured with two 6 bladed Rushton impellers and static air sparger.

15 The fermenter was cultured at 37°C and 100rpm with an airflow of 1.7l min^{-1} (1.7vvm) at pH 7.2 for 48 hours prior to using this primary seed stage to inoculate 40l of sterile SFG medium (7gl^{-1} glycerol).

20 This stage was cultured at 41°C and 55rpm (two Rushton turbines in a 50l fermenter) for 43 hours at an airflow of 40l min^{-1} .

Two litre portions of this culture were used to inoculate 38l of a sterile SFG medium in a 50l fermenter. This stage was cultured at 41°C and 20l min^{-1} airflow with control of dissolved oxygen at 70% of saturation by
25 cascade control of agitator speed.

After 13 hours of incubation the pH in each fermenter was adjusted over the course of 60 minutes to 8.5 by addition of 2M sodium hydroxide. Substrate (ferulic acid) was added as a single charge, dissolved in 5 10l of 0.5M sodium hydroxide at 41°C, with adjustment of this solution to pH 8.5 with 10M base. The correct concentration was verified by HPLC analysis, as was the subsequent course of reaction. Culture conditions were maintained as above.

10 Reaction of substrate to product was complete after between 40 and 51 hours.

The amounts of ferulic acid were varied, and the strength of the SFG final stage medium was adjusted according to substrate concentration:

Substrate concentration (gl ⁻¹)	Soyflour (Nutrisoy) (gl ⁻¹)	di-Potassium hydrogen orthophosphate (gl ⁻¹)	Magnesium Sulphate heptahydrate (gl ⁻¹)	Polypropylene glycol (ml)	Glycerol (gl ⁻¹)
18	5	1	1.64	0.2	7
25	8.6	1.7	2.8	.35	12
32	8.6	1.7	2.8	.35	12

15

3. Downstream purification

Extraction: At the conclusion of the biotransformation, the pH of the broth was adjusted to 6.7 +/- 0.1. It was then heat treated at 55°C for 5 20 minutes and then cooled to 30°C. Heat treated broth, 50l was then clarified either by centrifugation at -11,000 xg or more preferably by filtration using a filter aid, e.g.

Celite 512. The filter press used for clarification, a British Filters PA20M, is pre-coated with 10g of Celite per plate and 12gl⁻¹ of bodyfeed or admix added to the broth. The resulting filtrate was then ultrafiltered
5 through any suitable membrane, preferably one made of polysulphone and having a molecular weight cut-off of 10,000 Daltons.

To maximise recovery the ultrafiltrate retentate, 4l, was diafiltered three times with an equal volume of
10 water.

The combined ultrafiltrate and diafiltrate, 66l, were then adjusted to pH 6.6 +/- 0.1 and concentrated to approximately 50gl⁻¹ vanillin under reduced pressure using a pot still. The concentrate temperature was maintained
15 between 20 and 35°C at a vacuum of 1.3 to 26 KPa. On subsequent cooling of the final concentrate to 5-10°C in a suitable jacketed vessel, a crude vanillin product precipitated. After cooling at 5-10°C for not less than 3 hours the crude product was recovered by any suitable
20 means, but preferably filtration using a basket centrifuge (Broadbent 9 inch type 41) and dried in trays at 30-40°C and 80KPa.

Alternatively, the clarified broth may be concentrated to approximately 40gl⁻¹ at a higher
25 temperature suitably 30-35°C, and the resulting

concentrate, after adjustment of pH to 5.9, extracted with an equal volume of n-butyl acetate. After separation of the phases the rich solvent extract is concentrated to 320g l⁻¹ vanillin using a pot still and cooled, whereupon a precipitate of crude vanillin is formed which is recovered by vacuum filtration on a Nutsche filter. The filter cake can then be washed with 2 volumes of hexane to remove the n-butyl acetate and then dried in trays under vacuum, 80 KPa and 40°C.

10 The resulting dry material may be purified by either of the methods outlined under Purification below.

Purification

15 Dried product (554g, 53% vanillin) from initial downstream processing was charged into an extraction vessel of a carbon dioxide extraction rig. Pressure was raised to 20MPa and temperature set at 5°C. Liquid carbon dioxide rich in vanillin was piped to the evaporator, maintaining the above temperature and pressure conditions. In the evaporator the pressure was reduced to approximately 3MPa whereupon white crystals of vanillin precipitated. Temperature was maintained in the evaporator by circulation of a heat transfer medium in the evaporator jacket at 70°C. Vanillin (278.9g 95% yield) was recovered from the evaporator.

25

Further enhancement of product aroma may be achieved by washing the material with a solution of 4% polar solvent such as acetone or ethanol in a low polarity carrier such as hexane. Four volumes of wash are used
5 for every weight unit of product.

Instead of washing, the purified material may be treated in a fluidised-bed dryer. We used one produced by Sherwood Scientific, Cambridge, GB. Treatment at 55°C for 2 hours with a flow rate of CO₂ of 14.8-15.3L/sec
10 caused the organoleptic quality to be greatly improved by a method that is simple to use, acceptable as regards 'natural' and 'organic' specifications for flavours, and entails only minor (1-2%) losses consistent with normal handling operation losses. The colour of the product was
15 determined using a Minolta chromameter as explained above. It has an 'L' value of 99.5; and also an 'a' value (red-green hue) of 0.01, and a 'b' value (yellow-blue hue) of 2.84.

Fluidisation with air is also possible, but less
20 preferred than CO₂.

Fluidised bed treatment may also be used instead of the liquid CO₂ extraction method for the initial purification.

Another alternative is short-path distillation.
25 This may be applied to a semi-purified dry product, from

solvent extraction of concentrated broth. For example, the dry product is melted and fed continuously at 90-95°C into suitable short path distillation equipment, preferably one having a wiped surface evaporator, e.g.:

- 5 VKL 70 (41). With the melt at between 180 and 190°C and under a vacuum of between 1.5 and 2.0 KPa pure vanillin distills and is collected on a condenser operated at 90-95°C. 154.3g of vanillin was collected representing 64.6% of feedstock. Upon cooling, the distillate solidifies
- 10 and requires milling using a knife or flail mill.

Results of some runs are presented in Table 1 below:

Table 1: Production of vanillin using Zyl 926

Ferulic acid added	18	25	31-33
Conc. of vanillin formed (g/l)	10.8	13.8	17.95
Fermentation Time (h)	38.7	51.5	50.5
Residual Ferulic acid cons (g/l)	0.49	1.01	1.52
Volumetric Productivity (gV/L/H)	0.279	0.268	0.347
Yields of vanillin on ferulic acid supplied (% g/g) [% of theoretical yield]	62.17 [79.7%]	55.9 [71.7%]	56.17 [62,9%]